DD

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 48/00, C12N 15/11, 15/85, 15/86

A1

(11) International Publication Number: **WO 00/20040**

(43) International Publication Date: 13 April 2000 (13.04.00)

(21) International Application Number: PCT/US99/23489

(22) International Filing Date: 8 October 1999 (08.10.99)

(30) Priority Data:
09/169,446 8 October 1998 (08.10.98) US

(71) Applicant: UNIVERSITY OF MASSACHUSETTS [US/US]; One Beacon Street, 26th floor, Boston, MA 02108 (US).

(72) Inventors: GREEN, Michael, R.; 368 Green Street, Boylston, MA 01505 (US). WERSTUCK, Geoff; 18 Garden Avenue, Ancaster, Ontario L96 2J5 (CA).

(74) Agent: FASSE, J., Peter; Fish and Richardson P.C., 225 Franklin Street, Boston, MA 02110–2804 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CONTROLLING GENE EXPRESSION IN LIVING CELLS

(57) Abstract

Methods and compositions for controlling expression of a gene in a living cell are disclosed. In general, the methods include contacting the 5' untranslated region (5' UTR) of an RNA in the cell with a cell–permeable, small molecule. In some embodiments of the invention, the method includes providing an aptamer that binds specifically to the cell–permeable, small molecule; incorporating the aptamer into a region of a gene, which region encodes a 5' UTR of an RNA; and contacting the cell–permeable, small molecule with a cell that contains the gene. The cell–permeable, small molecule enters the cell and binds specifically to the aptamer sequence in the 5' UTR of RNA molecules transcribed from the gene. This binding specifically inhibits translation of the RNA molecules to which the cell–permeable, small molecule is bound, thereby controlling expression of the gene.

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

CONTROLLING GENE EXPRESSION IN LIVING CELLS

Field of the Invention

The invention relates to biochemistry, molecular biology, cell biology, medicine, and gene therapy.

Background of the Invention

A method commonly known as "*in vitro* selection" (Ellington et al., *Nature* **346**:818-822 (1990), "*in vitro* evolution" (Joyce, *Gene* **82**:83-87 (1989), or "SELEX" (Selective Evolution of Ligands by Evolution) Tuerk et al., *Science* **249**:505-510 (1990) allows the screening of large random pools of nucleic acid molecules for a particular functionality. This technique has been used to screen for functionalities such as binding to small organic molecules (Famulok et al., *Am. J. Chem. Soc.* **116**:1698-1706 (1994); Connell et al., *Biochemistry* **32**:5497-5502 (1994); Ellington et al., *Nature* **346**:818-822 (1990)), large proteins (Jellinek et al., *Proc. Natl. Acad. Sci. USA* **90**:11227-11231 (1993); Tuerk et al., *Proc. Natl. Acad. Sci. USA* **89**:6988-6992 (1992); Tuerk et al., *Gene* **137**:33-39 (1993); Schneider et al., *J. Mol. Biol.* **228**:862-869 (1992)); and the alteration or *de novo* generation of ribozymes (Liu et al., *Cell* **77**:1093-1100 (1994); Green et al., *Nature* **347**:406-408 (1990); Green et al., *Science* **258**:1910-1915 ((1992); Pun et al., *Biochemistry* **31**:3887-3895 (1992); Bartel et al., *Science* **261**:1411-1418 (1993). Functional molecules, known as "aptamers" (from "aptus," Latin for fit) are selected by column chromatography or any other technique of enrichment for the desired function.

For *in vitro* selection, a pool of oligonucleotides is synthesized with a completely random base sequence flanked by PCR primer binding sites. The pool is

subjected to the enrichment step, and then selected molecules are amplified in a PCR step. Up to 10^{15} different molecules, i.e., every possible permutation of an oligonucleotide containing a 25-base sequence, can be generated in this way and then screened simultaneously. Large numbers of random permutations of longer base sequences can be generated by carrying out the PCR step under mutagenic conditions (Lehman et al., *Nature* **361**:182-185 (1993); Beaudry et al., *Science* **257**:635-641 (1992)).

Summary of the Invention

We have discovered that aptamers incorporated into an RNA faithfully bind their ligand *in vivo*. Based on this discovery, the invention provides methods for controlling expression of a gene in a living cell. In general, the method includes contacting the 5′ untranslated region of an RNA in the cell with a cell-permeable, small molecule. In some embodiments of the invention, the method includes providing an aptamer that binds specifically to a cell permeable, small molecule; incorporating the aptamer into a region of a gene, which region encodes a 5′ untranslated region (5′ UTR) of an RNA; and contacting the cell-permeable, small molecule with a cell that contains the gene. The cell-permeable, small molecule enters the cell and binds specifically to the aptamer sequence in the 5′ UTR of RNA molecules transcribed from the gene. This binding specifically inhibits translation of the RNA molecules to which the cell-permeable, small molecule is bound, thereby controlling expression of the gene, e.g., by inhibiting or enhancing expression.

The gene whose expression is controlled can be an endogenous gene or a transgene. The cell can be a prokaryotic cell or a eukaryotic cell. In some

embodiments, the eukaryotic cell is a mammalian cell. The mammalian cell can be *in vivo*, e.g., in a human receiving gene therapy. The cell-permeable molecule can be administered to the mammal by any suitable route, e.g., topically, parenterally, orally, vaginally, or rectally.

The invention also provides a gene containing an aptamer sequence incorporated into a region of the gene that encodes a 5′ UTR of an RNA. The invention also provides a transgenic cell containing an aptamer incorporated into a region of a gene that encodes a 5′ UTR of an RNA. Preferably, the cell includes an RNA transcript containing the aptamer in the 5′ UTR of the RNA transcript. The cell can contain a cell-permeable, small molecule that binds specifically to the aptamer.

The invention also provides a bacterial resistance marker. The marker includes an aptamer sequence operably linked to a bacterial expression control sequence.

The invention also provides a method for determining whether a gene of interest is essential for the survival or growth of a cell. This method is useful in target validation studies. The method includes structurally disrupting or deleting an endogenous gene of interest in a cell; providing an aptamer that binds specifically to a cell-permeable, small molecule; incorporating the aptamer into a region of the gene of interest *in vitro*, which region encodes a 5′ untranslated region of an RNA, thereby producing a controllable gene of interest; introducing the controllable gene of interest into the cell, thereby producing a test cell; and contacting the cell-permeable, small molecule with the test cell, so that the cell-permeable, small molecule enters the test cell and controls expression of the controllable gene of interest.

As used herein, "cell-permeable, small molecule" means a molecule that permeates a living cell without killing the cell, and whose molecular mass is about 1,000 Daltons or less.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present application, including definitions will control. All publications, patents, and other references mentioned herein are incorporated by reference.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting. Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1 is a tobramycin-binding consensus aptamer nucleic acid sequence, with predicted secondary structure indicated.

Fig. 2 is a kanamycin A-binding consensus aptamer nucleic acid sequence, with predicted secondary structure indicated.

Figs. 3A-3E are growth curves of *E. coli* expressing antibiotic aptamers. Overnight cultures of BL-21 cells transformed with plasmids expressing RSETA, tob1, tob3, kan1, or kan3 were diluted 100-fold into medium containing the indicated concentration of aminoglycoside antibiotic. Optical density (660 nm) was measured at fixed intervals over 8 hours of growth at 37°C. Fig. 3A shows data on bacterial growth in the

absence of drug. Fig. 3B shows data on bacterial growth in the presence of 10 μM Kanamycin A. Fig. 3C shows bacterial growth in the presence of 10 μM Tobramycin. Fig. 3D shows growth in the presence of 20 μM Kanamycin A. Fig. 3E shows bacterial growth in the presence of 20 μM Tobramycin.

Fig. 4. is a histogram showing percent translation of mRNA in a wheat germ *in vitro* translation system containing 0 (RSETA) or 3 copies of the tob aptamer cloned into the 5' UTR of RSETA (tob3-RSETA) and 0, 30, or 60 μM tobramycin or kanamycin A. Protein products were analyzed by SDS-PAGE and quantitated by densitometry. For each transcript, translation in the absence of drug was set at 100%.

Fig. 5 is the chemical structure of Hoechst Dye H33258.

Fig. 6 is the chemical structure of Hoechst Dye H33342.

Fig. 7 is the nucleotide sequence and predicted secondary structure of H33258 aptamer H10, based upon the computer modeling program Mulfold. A Hoechst dye aptamer consensus sequence ($UUAN_{4-5}UCU$) was identified after 10 rounds of selection. The fixed primer binding regions are shown in plain print, selected bases are in bold, and the selected consensus sequence is indicated by outline print.

Fig. 8 is the nucleotide sequence and predicted secondary structure of H33258 aptamer H19, based upon the computer modeling program Mulfold.

Fig. 9 is a histogram summarizing data on the interaction of H10 and H19 aptamers with H33258, as indicated by percentage of total bound RNA eluted from an affinity column. Labeled aptamer (200,000 cpm of ^{32}P-UTP) was loaded onto a 0.25 ml H33258-SEPHAROSE™ column. Each column was then washed sequentially with 6 ml binding

buffer, 1 ml binding buffer containing 5 mM H33258, and 1 ml binding buffer containing 25 mM H33258. Fractions were collected and quantitated by scintillation counting.

Fig. 10. is a histogram summarizing SDS-PAGE densitometry data from *in vitro* translation experiments. RNA transcripts containing 0 (RSETA) or 2 copies of an H33258 aptamer (H2-RSETA) were translated in a wheat germ extract in the presence of ^{35}S-methionine and 0, 40 or 80 μM H33258. Protein products were subjected to SDS-PAGE and quantitated by densitometry. For each transcript, translation in the absence of drug was set at 100%.

Fig. 11 is a histogram summarizing data from *in vivo* expression experiments. H33258 aptamers H10 and H19 were cloned in tandem into the 5' UTR of a β-galactosidase reporter gene (SVβgal; Promega) to generate SVH2βgal. CHO cells were cotransfected with 1 μg SVβgal or SVH2βgal and 1 μg of a luciferase expression vector (pGL3). Transfected cells were grown in the presence of 0, 5, or 10 mM H33342. Twenty-four hours after transfection, cell extracts were prepared, and β-galactosidase and luciferase activities were determined.

Detailed Description

Providing an Aptamer

Techniques for *in vitro* selection of aptamers that bind specifically to a particular cell-permeable molecule, i.e., ligand, are known in the art. Those techniques can be employed routinely to obtain an essentially unlimited number of aptamers useful in the present invention. Examples of publications containing useful information on *in vitro* selection of aptamers include the following: Klug et al., *Molecular Biology Reports* **20**:97-107 (1994); Wallis et al., *Chem. Biol.* **2**:543-552 (1995); Ellington, *Curr. Biol.* **4**:427-429 (1994); Lato et al., *Chem. Biol.* **2**:291-303 (1995); Conrad

et al., *Mol. Div.* 1:69-78 (1995); and Uphoff et al., *Curr. Opin. Struct. Biol.* 6:281-287 (1996).

The basic steps in conventional *in vitro* selection of an aptamer are as follows. A random DNA pool is synthesized, i.e., a pool of DNA molecules having random nucleotide sequences. The random DNA pool is transcribed to produce a random RNA pool. The RNA pool is subjected to affinity chromatography. RNA molecules that bind specifically to the immobilized ligand are collected and reverse-transcribed into cDNA and amplified by PCR. The PCR-amplified products are transcribed into RNA. The process is repeated for as many cycles as necessary to yield a population of nucleic acid molecules that bind to the ligand with the desired affinity (and specificity). Individual nucleic acid molecules from the selected population are cloned and sequenced using conventional recombinant DNA technology. Such technology is described in numerous references, e.g., Sambrook et al., *Molecular Cloning - A Laboratory Manual* (2nd ed.), Cold Spring Harbor Laboratory Press (1989).

For any given cell-permeable, small molecule (ligand), a potentially large number of different, useful aptamers can be isolated by one of ordinary skill in the art, using conventional techniques, without undue experimentation. The aptamers are empirically selected from a random pool of nucleic acid molecules by predictable selection methods. Therefore, it is not necessary to know in advance of the selection process what the nucleotide sequence of the aptamer will be.

The optimal length of the random nucleotide sequence in the aptamer length will vary, depending on factors including the size and shape of the ligand. Preferably, the length of an aptamer used in this invention is between 10 and 200 nucleotides. More preferably, the length is between 20 and 100 nucleotides.

Among the numerous aptamer-ligand pairs useful in this invention, aptamer-ligand binding affinities can vary widely. In general, the affinity is high enough to provide effective control of gene expression, but not so high as to make the aptamer-ligand binding effectively irreversible. Determination of whether a particular aptamer-ligand pair displays a suitable binding affinity is within ordinary skill in the art.

Incorporating the Aptamer

After isolation of an aptamer that binds the cell-permeable molecule (ligand) with suitable affinity and specificity, the aptamer is incorporated into the 5′ UTR of a gene whose expression is to be controlled. The incorporation can be carried out, without undue experimentation, using conventional recombinant DNA technology.

The gene whose expression is to be controlled can be an endogenous gene or a transgene. When the gene is an endogenous gene, the aptamer can be incorporated into the 5′ UTR by known techniques of gene targeting, i.e., homologous recombination. When the gene is a transgene, preferably the aptamer is incorporated into the 5′ UTR by *in vitro* manipulation of the transgene or a DNA vector containing the transgene.

A gene controlled according to this invention can be in a prokaryote or a eukaryote. The gene can be in an episome, e.g., a plasmid, or a genome, e.g., a mammalian chromosome. A transgene or gene targeting vector can be introduced into the living cell (that will be contacted with the cell permeable molecule), or a progenitor of the cell, by any suitable means. The suitable means will depend, at least in part, on the identity of the living cell. This is illustrated by the following non-limiting examples. If the living cell is a yeast cell, the

transgene or gene targeting vector can be electroporated directly into the yeast cell or a progenitor of the yeast cell. If the cell is in a transgenic plant, the transgene or gene targeting vector can be introduced into regenerable plant tissue culture cells by electroporation, ti-plasmid, or microparticle bombardment. If the living cell is a cell in a transgenic, non-human mammal, the transgene or gene targeting vector can be microinjected into an embryonic cell that is used to produce the non-human mammal. If the cell is *in vivo* in a human receiving gene therapy, the transgene or gene targeting vector can be introduced into target cells of the human by any suitable gene therapy technique, e.g., a viral vector or injection of naked DNA.

Cell-Permeable, Small Molecule

There is wide latitude in the choice of the cell-permeable, small molecule used in this invention. The cell-permeable, small molecule must bind an aptamer with suitable affinity and specificity. Whether a molecule will bind to an aptamer with suitable affinity and specificity depends on factors including molecular size, shape and charge. Those of skill in the art will appreciate that the cell-permeable molecule can be chosen first, and then used for *in vitro* selection of an aptamer that binds to it. Choosing a cell-permeable, small molecule that is suitable for use in *in vitro* selection of an aptamer is within ordinary skill in the art.

Preferably, the cell-permeable, small molecule displays low toxicity, so that unwanted biological side effects are minimized. When the cell containing the gene to be controlled is *in vivo*, the cell-permeable, small molecule is chosen to have an *in vivo* persistence

sufficient to allow an effective amount of the cell permeable, small molecule to reach and enter the cell.

In some embodiments of the invention the cell-permeable, small molecule is a drug previously approved for use in humans. Using an approved drug can be advantageous, because information on safety, side effects, dosage, route of administration, pharmacokinetics, metabolism, clearance and other useful information is available. Preferred drugs are those that display mild pharmacological activities and minimal side effects.

It is not necessary, however, for the cell-permeable, small molecule to be a drug. In preferred embodiments of the invention, the cell-permeable, small molecule is pharmacologically inert (except for its activity in binding the aptamer according to this invention). Preferably, the cell-permeable, small molecule is an organic compound. The design and synthesis of small, organic, cell-permeable molecules useful in this invention are described, for example, in Amara et al., *Proc. Natl. Acad. Sci. USA* **94**:10618-10623 (1997); and Keenan et al., *Bioorganic & Medicinal Chemistry* **6**:1309-1335 (1998).

Formulating and Administering
the Cell-Permeable, Small Molecule

The cell-permeable, small molecule can be formulated, individually or in combination, into pharmaceutical compositions by admixture with pharmaceutically acceptable nontoxic excipients and carriers. Such compositions can be prepared for use in parenteral administration, particularly in the form of liquid solutions or suspensions; for oral administration, particularly in the form of liquid, tablets or capsules;

or intranasally, particularly in the form of powders, nasal drops, or aerosols.

The composition can be administered conveniently in unit dosage form and can be prepared by any of the methods known in the art. Such methods are described, for example, in *Remington's Pharmaceutical Sciences* (Mack Pub. Co., Easton, Pa., 1980).

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active compound, the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Injectable depot forms are made by forming microencapsulated matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, 3) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The active compounds can also be in micro-encapsulated form with one or more excipients as noted above. In solid dosage forms the active compound may be admixed with at least one inert diluent such as sucrose, lactose or starch. Such dosage forms may also comprise, as is normal practice, additional substances other than inert diluents, e.g., tableting lubricants and other tableting aids such a magnesium stearate and microcrystalline cellulose. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

Target Validation

The present invention can be used in "target validation" studies. The goal of target validation is to determine whether a particular gene is essential for the survival or growth of a particular type of cell, e.g., a bacterial pathogen. If a gene of interest is an essential gene, it (or its expression product) constitutes a potential drug target, which can be used for drug screening or rational drug design.

Target validation technology has previously relied on a conventional gene "knockout" approach. See, e.g., Arigoni et al., *Nature Biotechnology* **16**:851-856 (1998). A disadvantage of the conventional gene knockout approach

is that the gene is either present or absent, i.e., intermediate levels of expression of the gene of interest are not evaluated.

The present invention advantageously allows measurement of the effect of intermediate levels of expression of the gene of interest. For example, a 50% reduction in expression of an essential gene might be sufficient to cause the death of a microbial pathogen. Such information, now can be obtained readily through the use of this invention.

Examples

The invention is further illustrated by the following examples. The examples are provided for illustration purposes only, and are not to be construed as limiting the scope or content of the invention in any way.

We demonstrated that bacteria expressing an aptamer to an aminoglycoside antibiotic are resistant to the cognate drug. This indicated that a small molecule-aptamer interaction occurred *in vivo*. To regulate gene expression, aminoglycoside aptamers were inserted into the 5′ UTR of an mRNA, whose *in vitro* translation then became repressible by drug addition. To determine if a similar approach could work *in vivo*, we derived RNA aptamers for cell-permeable Hoechst dyes and inserted them into the 5′UTR of a β-galactosidase reporter gene. Following transfection into mammalian cells, expression of the reporter gene was specifically inhibited by drug addition.

An initial 70 nucleotide RNA pool containing 31 random nucleotides was constructed essentially as described by Singh et al., *Science* **268**:1173 (1995). Tobramycin or kanamycin A were covalently linked to CNBr-activated Sepharose 4B. Aminoglycosides (2 mmoles) were

dissolved in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), then mixed with CNBr-activated Sepharose 4B (preswollen in 1 mM HCl) and incubated at 4°C for 12-16 hours. The resin was then washed and remaining active groups blocked with 0.2 M glycine. Pre-selection columns were prepared with glycine alone.

The RNA pool (approximately 10^{15} individual sequences) was dissolved in selection buffer (50 mM Tris, pH 8.3, 250 mM KCl, 2 mM $MgCl_2$) heated to 80°C for 3 minutes and cooled to room temperature. RNA was then loaded onto a pre-selection column (0.25 ml glycine-Sepharose) to remove RNAs that bound to the column, the resin, or glycine. Non-binding RNAs were eluted with two column volumes of selection buffer and immediately loaded onto a 0.5 ml aminoglycoside-Sepharose column. Columns were washed with 10 column volumes of selection buffer (selection rounds 1-5), 10 column volumes buffer with 5 mM competitor aminoglycoside (rounds 6-9), or 10 column volumes buffer with 10 mM competitor (rounds 10-14). The competitor aminoglycoside for tobramycin aptamer selection was kanamycin A and vice versa. In each round, bound RNA was eluted with 5 mM of the cognate aminoglycoside.

Eluted RNA was RT-PCR amplified using flanking primers. The PCR products were transcribed into RNA with T7 RNA polymerase and purified by polyacrylamide gel electrophoresis. Pools were subcloned into the plasmid pBlueScript (Stratagene) and sequenced after rounds 10, 12, and 14. Isolation of H33258 aptamers was carried out in a similar manner, with the following exceptions. H33258 was covalently linked to epoxy-activated Sepharose 6B. The ligand solution was mixed at 37°C for 16 hours. The resin was then washed and excess active groups were blocked with 1 M ethanolamine (pH 10). Pre-selection columns were prepared with ethanolamine alone. H33258

selection buffer contained 50 mM Tris pH 7.3, 200 mM KCl, 2mM $MgCl_2$.

In selection rounds 1-6, columns were washed with 20 column volumes of selection buffer and eluted with 2 column volumes of 10 mM H33258. In selection rounds 7-10, columns were washed with 20 column volumes buffer and 20 column volumes 10 mM benzimidazolepropionic acid (in selection buffer) before elution.

Fig. 1A shows the consensus sequences and secondary structures of our kanamycin A and tobramycin aptamers, which differ at only two of fourteen bases. As an initial test for the ability of these aptamers to function *in vivo*, we asked whether following expression in *E. coli* the aptamer would sequester the cognate antibiotic thereby conferring a specific drug-resistant phenotype. Toward this end, one or three copies of the kanamycin A (kan) or the tobramycin (tob) aptamer were cloned into the T7 RNA polymerase-driven expression vector pRSETA (Invitrogen), and transformed into a bacterial strain containing an IPTG-inducible T7 RNA polymerase. Bacterial strains were grown in liquid culture overnight and then diluted into antibiotic-containing medium. In the absence of drug, bacterial strains expressing no aptamer (bl-RSETA), the kanamycin aptamer (bl-kan1), or the tobramycin aptamer (bl-tobl) grew similarly (Fig. 3A). In the presence of 10mM kanamycin A, bl-kanl grew to saturation, whereas growth of bl-RSETA and bl-tobl was negligible (Fig. 3B). In the presence of 10 mM tobramycin, bl-tobl grew to saturation, and bl-kanl also grew to a sub-saturating level (Figure 3C). The partial-resistance of bl-kanl to tobramycin (our unpublished data). Figures 3D and 3E show that increasing the number of aptamers in the expression vector from one to three, enhanced growth in the presence of antibiotic. None of the strains exhibited increased

resistance to unrelated antibiotics. Collectively, these results indicate that a specific drug-resistant phenotype can be conferred by expression of an aminoglycoside aptamer, demonstrating the occurrence and specificity of a small molecule-aptamer interaction *in vivo*.

Based upon the *in vitro* results, we next designed experiments to investigate whether small molecule aptamers could be used to regulate gene expression *in vivo*. We designed these experiments in view of the fact that eukaryotic translation initiation typically involves 5′-to-3′ scanning from the 5′-m^7G cap to the start codon (Kozak, *Ann. Rev. Cell Biol.* 8:197 (1992); Sachs et al., *Cell* 89:831 (1997)), and binding of a protein between the cap and start codon can repress translation, presumably by blocking either scanning or the ribosome-mRNA interaction (Stripecke et al., *Mol. Cell. Biol.* **14**:5898 (1994); Paraskeva et al., *Proc. Natl. Acad. Sci. USA* **95**:951 (1998)). These considerations prompted us to test whether the presence of a small molecule-aptamer complex within the 5′ UTR would repress translation in an analogous fashion.

A test mRNA was constructed containing three copies of the tob aptamer inserted in the 5′ UTR of RSETA (tob3-RSETA). *In vitro* translation reactions were performed in the presence of 0, 30 or 60 μM tobramycin or kanamycin A.

In vitro transcription reactions contained 5 μg pRSETA (or RSET derivative), 0.5 mM $m^7G(5')G$, 0.5 mM ATP, CTP, UTP, 0.05 mM GTP, 10 mM DTT and 40 U T7 RNA polymerase in 50 μl of a solution of 40 mM Tris-HCl pH 7.5, 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl. Following incubation for 1 hour at 37°C, RNA was purified by phenol:chloroform extraction, ethanol precipitation and resuspended in 30 μl H_2O. Translation reactions were carried out in 10 μl containing 5 μl wheat germ extract,

0.8 μl 1 mM amino acid mixture (minus methionine), 2 μl of RNA transcript (described above), 0.5 μl [^{35}S]methionine (1200 Ci/mmole) and 0-80 μM drug. Reactions were incubated at 25°C for 15 minutes and terminated by addition of 2X sample loading buffer. Translation products were separated by electrophoresis on an 18% polyacrylamide gel, visualized by autoradiography, and quantitated by densitometry.

Translation of the control RSETA mRNA was unaffected by all concentrations of tobramycin or kanamycin tested. Addition of tobramycin inhibited *in vitro* translation of the tob3-RSETA mRNA in a dose-dependent fashion (Fig. 4). *In vitro* translation of the tob3-RSETA mRNA was not inhibited by comparable concentrations of kanamycin A, which is not recognized by the tob aptamer.

Our results indicated that small molecule-aptamer interactions occur faithfully *in vivo* (Figs. 3A-3E). The results summarized in Fig. 4 showed that in a cell-free system a small molecule can be used to regulate translation through a cis-acting aptamer. We therefore reconfigured the system for regulating gene expression *in vivo*. Because aminoglycosides were known to be relatively impermeable to the plasma membrane, to be cytotoxic, and at elevated concentrations to have a general inhibitory effect on translation, we elected to use a different cell-permeable small molecule as the translation regulator.

We chose the Hoechst dye 33258 (H33258) and the closely related drug H33342 (Figs. 5 and 6), because they were known to be relatively non-toxic and cell-permeable (Uphoff et al., *Curr. Opin. Struct. Biol.* 6:281 (1996)). We isolated RNA aptamers that bound specifically to H33258 by affinity chromatography on a column containing H33258 covalently attached to an epoxy-activated

sepharose resin through a single hydroxyl group. Figs. 7 and 8 show the sequences and secondary structures of two of these aptamers, H10 and H19, isolated after 10 rounds of selection. H10 and H19 bound to an H33258 affinity-column and required a relatively high concentration (25mM) of free H33258 for elution (Fig. 9). H10 and H19 bound H33258 and the closely related H33342 comparably (data not shown).

To demonstrate that the H33258-aptamer could be used to regulate translation, one copy of H10 and H19 were inserted in tandem into the 5' UTR of RSETA. Addition of H33258 inhibited *in vitro* translation of H2-RSETA, but not the control RSETA, in a dose-dependent fashion (Fig. 10).

To test whether this small molecule-aptamer interaction could be used to control gene expression *in vivo*, one copy of H10 and H'9 were inserted into the 5'UTR of a mammalian β-galactosidase expression plasmid SVβGal (Promega), generating the construct SVH2βgal. CHO cells were cotransfected with SVH2βGal or as a control the parental vector, SVβGal, and a luciferase reporter gene to provide an internal control. Following transfection, cells were grown for 24 hours in the presence of 0, 5 or 10 μM H33342 and analyzed for β-galactosidase and luciferase activities. In these experiments, H33342, rather than H33258, was used because it is approximately ten-fold more cell-permeable.

In the absence of drug, two H33258 aptamers in the 5'UTR had no effect on gene expression (compare SVβgal and SVH2βgal)(Fig. 11). This was consistent with the *in vitro* translation data shown in Fig. 10. Expression of the luciferase reporter (Figure 11) and the parental expression vector SVβGal (data not shown) were not inhibited by 0,5 or 10 uM H33342. H33342 reduced β-galactosidase activity from SVH2βGal greater than 90% in

a dose-dependent fashion. These results indicated that inhibition by H33342 is dependent upon the presence of an appropriate RNA aptamer in the 5'UTR, and that the small molecule-aptamer translation switch works both *in vitro* and *in vivo*.

H33258 aptamers, H10 and H19, were cloned in tandem into the 5' UTR of a β-galactosidase reporter gene (SVβgal, Promega) to generate SVH2βgal. CHO cells were cotransfected with 1 μg SVβgal or SVH2βgal and 1 μg of a luciferase expression vector (pGL3). Transfected cells were grown in the presence of 0, 5 or 10 mM H33342. 24 hours post-transfection cell extracts were prepared and β-galactosidase and luciferase activities were determined.

Other embodiments are within the following claims.

1. A transgenic cell comprising an aptamer incorporated into a region of a gene that encodes a 5' untranslated region of an RNA.

2. The cell of claim 1, further comprising an RNA transcript, wherein the aptamer is incorporated into the 5' untranslated region of the RNA transcript.

3. The cell of claim 2, further comprising a cell-permeable, small molecule that binds specifically to the aptamer.

4. A bacterial resistance marker comprising an aptamer sequence operably linked to a bacterial expression control sequence.

5. A method for controlling expression of a gene, the method comprising:

providing an aptamer that binds specifically to a cell-permeable, small molecule;

incorporating the aptamer into a region of a gene that encodes a 5' untranslated region of an RNA;

contacting the cell-permeable, small molecule with a cell that contains the gene, so that the cell-permeable, small molecule enters the cell and controls expression of the gene.

6. The cell of claim 3 or the method of claim 5, wherein the cell-permeable, small molecule binds specifically to the aptamer sequence in the 5' untranslated region of RNA transcribed from the gene.

7. The cell of claim 1 or the method of claim 5, wherein the gene is an endogenous gene.

8. The method of claim 5, wherein the gene is a transgene.

9. The cell of claim 1 or the method of claim 5, wherein the cell is a prokaryotic cell.

10. The cell of claim 1 or the method of claim 5, wherein the cell is a eukaryotic cell.

11. The cell or method of claim 10, wherein the eukaryotic cell is a mammalian cell.

12. The cell or method of claim 11, wherein the mammalian cell is *in vivo*.

13. The method of claim 12, further comprising administering the cell-permeable, small molecule to the mammal topically, parenterally, orally, vaginally, or rectally.

14. The cell of claim 3 or the method of claim 5, wherein the cell-permeable, small molecule is an organic compound.

15. A gene comprising an aptamer sequence incorporated into a region of the gene that encodes a 5' untranslated region of an RNA.

16. A method for determining whether a gene of interest is essential for the survival or growth of a cell, the method comprising:

structurally disrupting or deleting an endogenous gene of interest in the cell;

providing an aptamer that binds specifically to a cell-permeable, small molecule;

incorporating the aptamer into a region of the gene of interest *in vitro*, which region encodes a 5' untranslated region of an RNA, thereby producing a controllable gene of interest;

introducing the controllable gene of interest into the cell, thereby producing a test cell; and

contacting the cell-permeable, small molecule with the test cell, so that the cell-permeable, small molecule enters the test cell and controls expression of the controllable gene of interest to determine whether the gene of interest is essential.

17. A method for controlling the expression of a gene in a living cell, the method comprising contacting the 5' untranslated region of an RNA in the cell with a cell- permeable, small molecule.

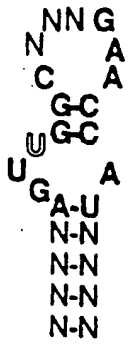


FIG. 1

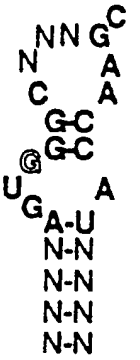


FIG. 2

FIG. 4

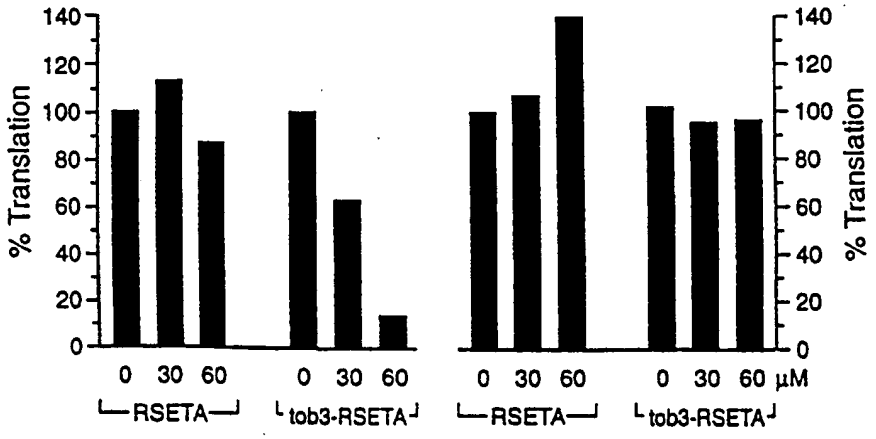


FIG. 3A

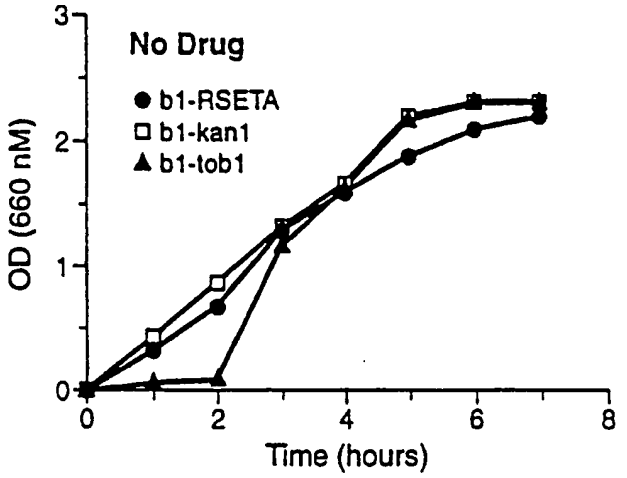


FIG. 3B

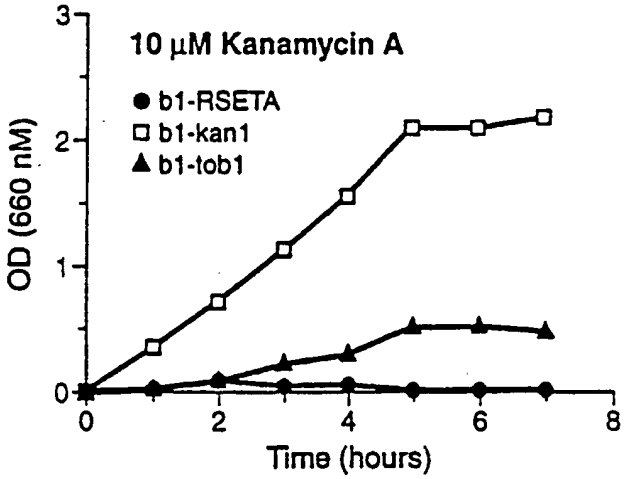


FIG. 3C

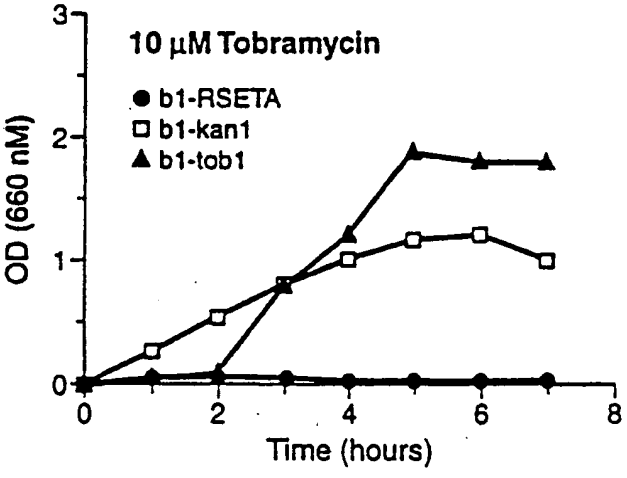


FIG. 3D

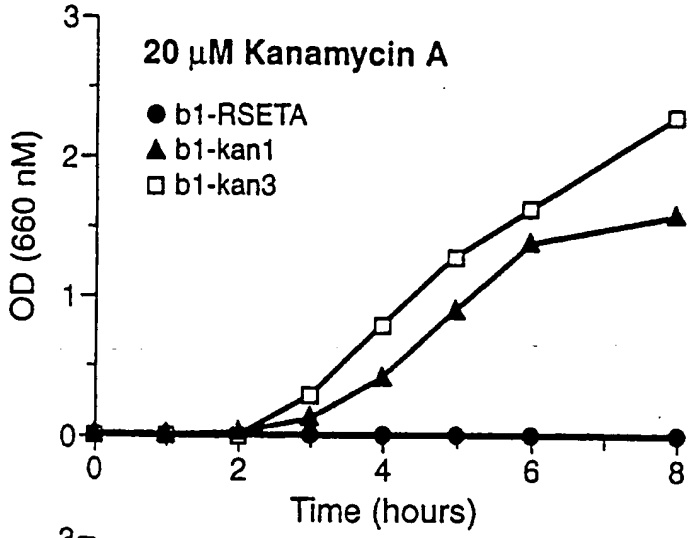
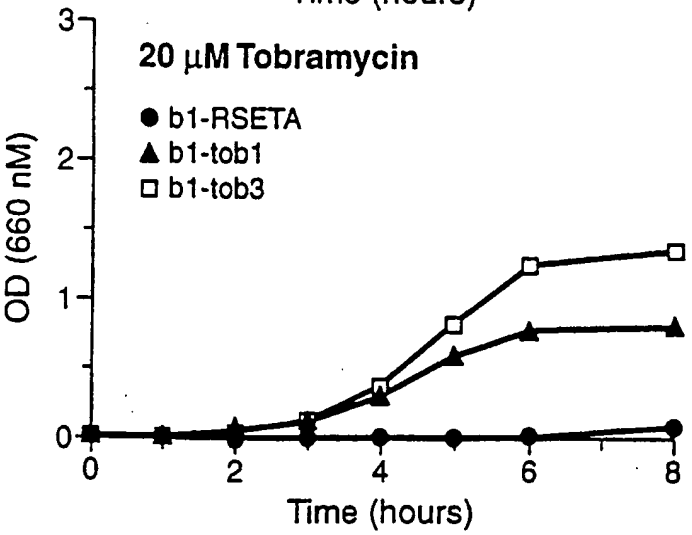

20 μM Kanamycin A
● b1-RSETA
▲ b1-kan1
□ b1-kan3
OD (660 nM)
Time (hours)
0
1
2
3
0
2
4
6
8

FIG. 3E


20 μM Tobramycin
● b1-RSETA
▲ b1-tob1
□ b1-tob3
OD (660 nM)
Time (hours)
0
1
2
3
0
2
4
6
8

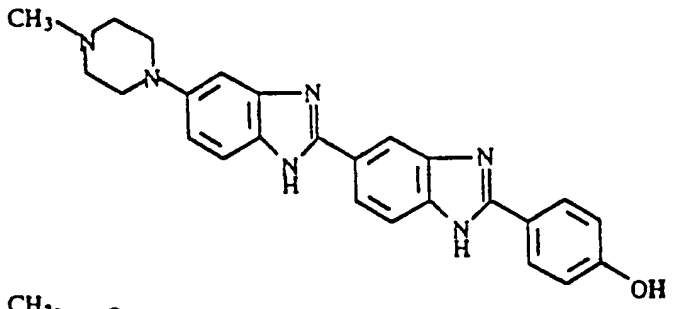


FIG. 5

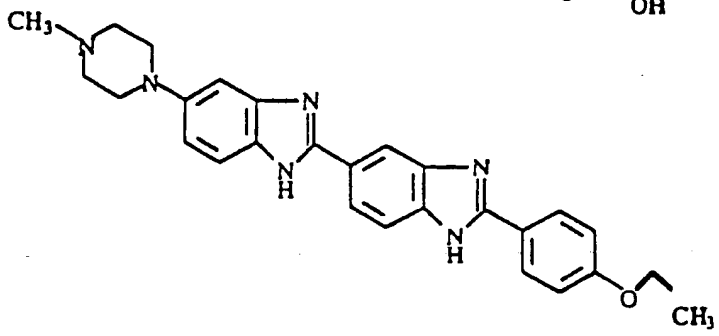


FIG. 6

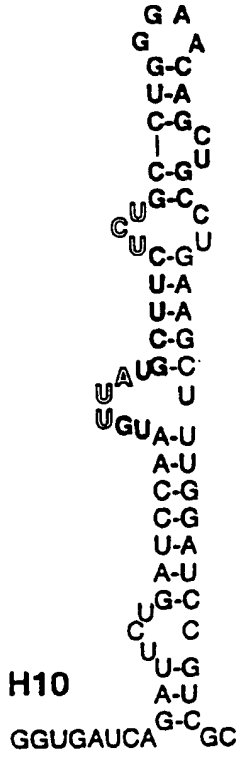


FIG. 7

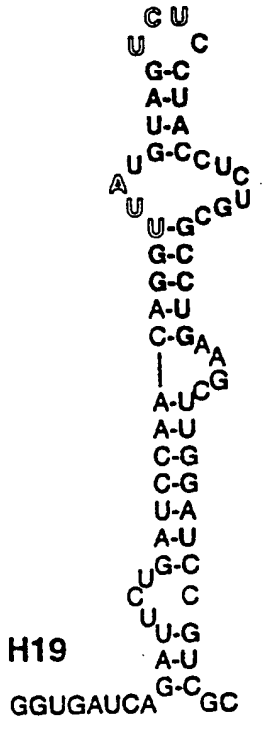


FIG. 8

FIG. 9

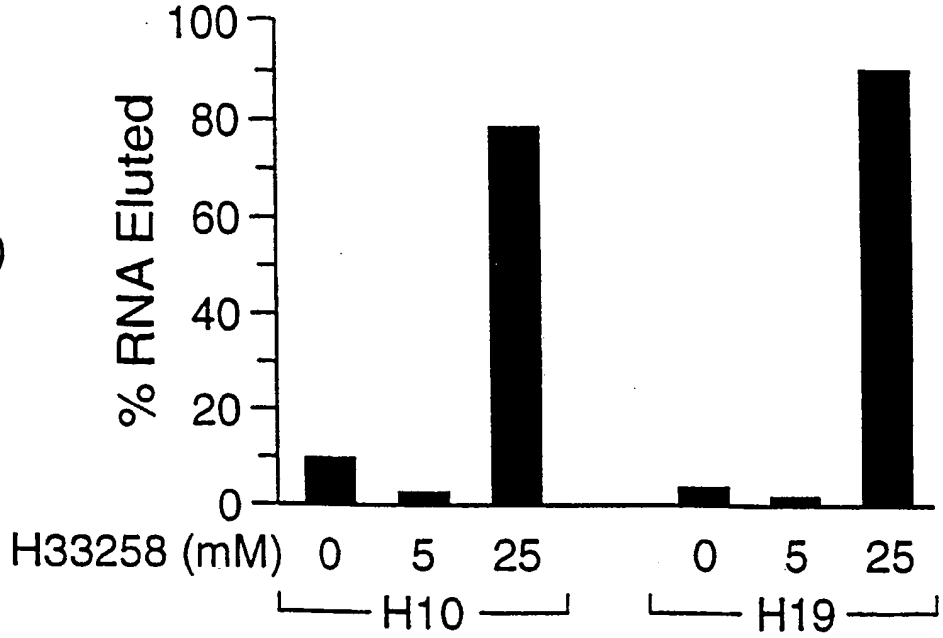

100
80
60
40
20
0
% RNA Eluted
H33258 (mM) 0 5 25 0 5 25
H10
H19

FIG. 10

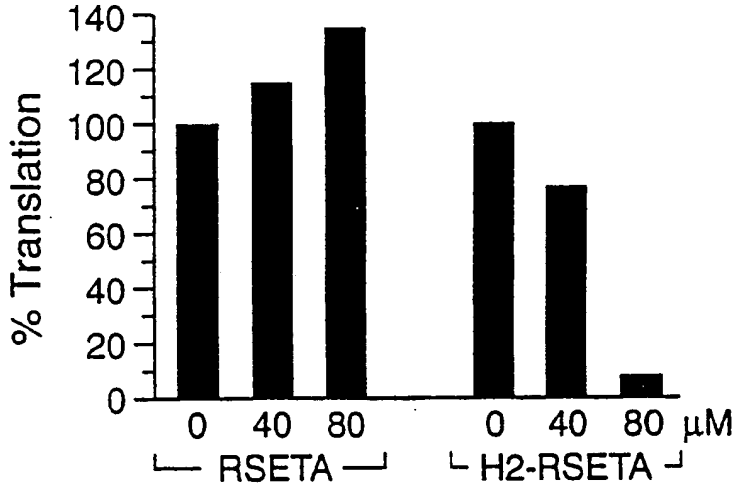
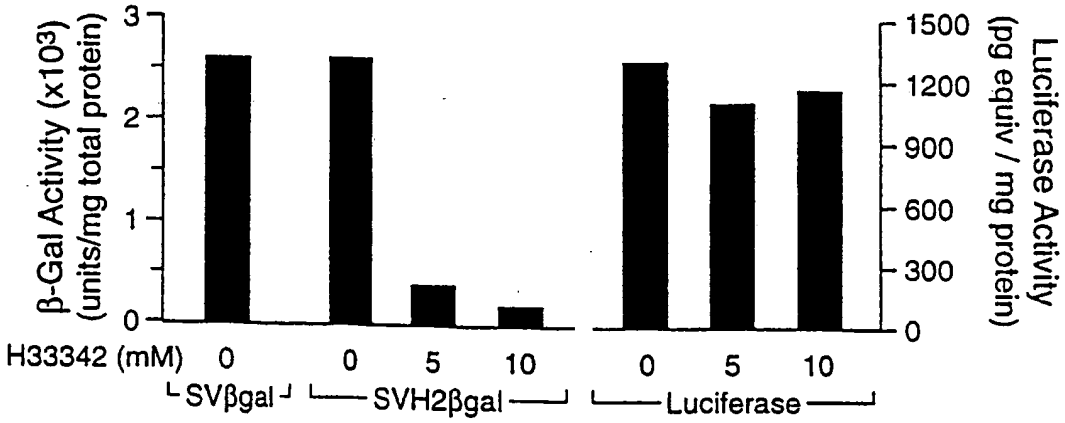

140
120
100
80
60
40
20
0
% Translation
0 40 80 0 40 80 µM
RSETA
H2-RSETA

FIG. 11


β-Gal Activity (x10³) (units/mg total protein)
3
2
1
0
1500
1200
900
600
300
0
Luciferase Activity (pg equiv / mg protein)
H33342 (mM) 0 0 5 10 0 5 10
SVβgal
SVH2βgal
Luciferase

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23489

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 48/00; C12N 15/11, 15/85, 15/86
US CL :435/325, 455; 514/44; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/325, 455; 514/44; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ARIGONI et al. A genome-based approach for the identification of essential bacterial genes. Nature Biotechnology. September 1998. Vol. 16, pages 851-856, entire document.	1-17
Y	ELLINGTON, A.D. Aptamers achieve the desired recognition: In vitro selection procedures can generate RNA molecules, known as aptamers, that bind pre-determined ligands with an affinity and selectivity comparable to highly evolved protein molecules. Current Biology. Vol. 1994, 4, No. 5, pages 427-429, entire document.	1-17
Y	ELLINGTON et al. In vitro selection of RNA molecules that bind specific ligands. Nature. 30 August 1990, Vol. 346, pages 818-822, entire document.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 DECEMBER 1999	Date of mailing of the international search report 07 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE-MARIE BAKER, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23489

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GOOD et al. Expression of small, therapeutic RNAs in human cell nuclei. Gene Therapy. 1997, Vol. 4, pages 45-54, entire document.	1-17
Y	WANG et al. Specific binding of aminoglycoside antibiotics to RNA. Chemistry & Biology. May 1995, Vol. 2, No. 5, pages 281-290, entire document.	1-17
Y,P	WERSTUCK et al. Controlling gene expression in living cells through small molecule-RNA interactions. Science. 09 October 1998, Vol. 282, pages 296-298, entire document.	1-17
Y	GOLD, L. Oligonucleotides as research, diagnostic, and therapeutic agents. J. Biol. Chem. 09 June 1995, Vol. 270, No. 23, pages 13581-13584, entire document.	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23489

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST
Dialog (file: medicine)
search terms: SELEX, aptamer, RNA, gene(w)regulation, expression, translation, inhibit?, repress?, UTR, aminoglycoside

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.